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STOMATA SIZE IN RELATION TO PLOIDY LEVEL IN NORTH AMERICAN HAWTHORNS (*CRATAEGUS*, ROSACEAE)

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Abstract

The impacts of ploidy level changes on plant physiology and ecology present interesting avenues of research, and many questions remain unanswered. Here, we examine the connections between cytotype, taxon, stomata characteristics, and environmental variables in black-fruited hawthorns of the Pacific Northwest (*Crataegus* ser. *Douglasianae*; Maleae, Amygdaloideae, Rosaceae). We explore the extent to which stomatal measurements can be used to predict ploidy level and how differences in ploidy level and stomata characteristics relate to geographic distributions. We sampled trees from across the geographic ranges of the putative sister taxa *Crataegus suksdorfii* (Sarg.) Kruschke (diploids and autotriploids) and *C. douglasii* Lindl. (tetraploids). We found that stomata differed between the two species, with tetraploid *C. douglasii* having larger average stomata sizes than diploid and triploid *C. suksdorfii*. We also obtained climatological and elevation data for the sites at which these samples were collected, and examined the associations between taxon, ploidy level, stomatal size and density, elevation, and environmental parameters. Our analyses indicate positive associations between stomatal size and latitude, and between ploidy level and elevation. Negative associations were found between temperature and precipitation variables and both ploidy level and stomatal size, particularly for the fall and winter quarters. There appeared to be no significant association between stomatal density and any of the environmental variables. Tetraploid *C. douglasii* occupied a wider range of environmental conditions than did either the diploids or the autotriploids.

Key Words: Agamospermy, climate, *Crataegus douglasii*, *Crataegus gaylussacia*, *Crataegus suksdorfii*, geographical parthenogenesis, polyploidy, stomata.

Determining the causal factors behind species distributions is a fundamental area of study in ecology (Krebs 2001). The realized range of an individual species can be affected by abiotic factors such as climate, biotic interactions such as competition, or a more complex interplay of environmental factors (Angert and Schemske 2005). Species ranges vary dramatically in area, and even closely related species can have very divergent range sizes (Brown et al. 1996). For some close relatives, differences in cytotype may be the underlying cause of range size differences; polyploidy in one species could lead to an ability to tolerate a greater or different range of conditions, and therefore facilitate a wider or novel distribution, compared to a diploid congener (Levin 1983; Krebs 2001; Parisod et al. 2010; Ramsey 2011). A polyploidization event may present opportunities in terms of range expansion, including a possible increase in genetic diversity, and a decrease in barriers to self-fertilization (Angert and Schemske 2005, Lowry and Lester 2006). In addition, polyploids have been found to have a number of advantages over their diploid relatives, including larger seed sizes, more vigorous seedlings, increased resistance to pathogens and pests, and higher tolerances for poor soils and drought conditions (Levin 1983; Brown et al. 1996). These differences can translate into divergent ecological tolerances and geographic distributions (Lewis 1980; Lowry and Lester 2006; Ramsey 2011; McIntyre 2012). Although differences between cytotypes in range sizes and environmental factors are not universal (Martin and Husband 2009), some rapid divergence after polyploidization is theoretically necessary for the new polyploid population to avoid direct competition with its progenitor, thereby allowing for its persistence (Coyne and Orr 2004; McIntyre 2012). However, the connections between polyploidy and observed ecological differentiation remain poorly understood (Li et al. 1996; Ramsey 2011).

Polyploidy is frequently associated with gametophytic apomixis, notably in *Ranunculus* L. species (Hörandl 2008) and in many Asteraceae, Poaceae, and Rosaceae (Nogler 1984). In these cases the wider range of apomicts may be related not only to polyploidy but also to the ability to set seed in the absence of other conspecific individuals (Hörandl 2006; Lo et al. 2013).

Investigations of the distribution and frequency of polyploidy typically depend on the availability of tissue from which chromosome counts can be made or, increasingly, from which stainable, intact nuclei can be extracted and passed through a flow cytometer. Neither of these approaches offers much chance of success with existing specimens from museum collections. Taxonomic recognition of major morphological differences between ploidy levels may be sufficient in some cases, as in the contrast between diploid hawthorns, all of which have 20 stamens per flower, while almost all tetraploid hawthorns have 10 stamens per flower (Talent and Dickinson 2005). Unfortunately, this pattern is confounded by intraspecific variation in ploidy level (e.g., *Crataegus suksdorfii* [Sarg.] Kruschke [Dickinson et al. 2008]), and morphological variation among closely related tetraploids (e.g., *C. crus-galli* L. *sensu* lato [Dickinson and Phipps 1986]). In addition, flowering traits may not always be visible on herbarium specimens.

Considering these issues, researchers may seek proxies for direct measurements of chromosome number or nuclear DNA content. Cell size is one such proxy, since polyploids typically have larger cells than diploids (Stebbins 1950; Levin 1983; Otto 2007; Beaulieu et al. 2008). In plants, the size of pollen grains and of stomatal guard cell pairs may both be accessible with herbarium material and work on a variety of taxa, including *Crataegus* (Marshall 1978) and other Rosaceae (Joly and Bruneau 2007), has shown how these traits can be used to predict ploidy level in herbarium specimens (Buechler 2000; Joachimiak and Grabowska-Joachimiak 2000; Saltonstall et al. 2007; Chen et al. 2009). Relatively simple methods for light microscopy may have the potential to reveal the ploidy level of long dead individuals from which herbarium specimens of historical interest were collected. However, in hawthorns the variation in pollen grain size within ploidy levels is so great that, while overall size increases with nuclear DNA content measured in leaves, no prediction from pollen measurements to ploidy level is possible; only stainability differentiates the pollen of diploids (highly stainable) from that of polyploids (Dickinson, unpublished data).

Importantly, features such as stomatal size and density have been recognized as varying not only with ploidy level, but also with environmental factors, such as temperature and water availability (Wang and Clarke 1993). The links between cytotype, stomata, and environmental variables have important implications for the realized ranges of plant species, and could help explain the differences in the breadth and characteristics of habitats occupied by congeners. Here, we investigate the way in which ploidy level, stomatal dimensions, and stomatal density vary with each other, and in relation to geographic distribution and the environment, as indexed by latitude, elevation, and climate parameters. We ask the following questions: (1) does size or some other readily measurable characteristic of cells with a fixed ontogenetic trajectory like stomata vary with ploidy level in such a way that this parameter can be used to predict ploidy level?, (2) does size or some other characteristic of stomata vary between taxa even when ploidy level is kept constant?, and finally (3) is cell size or other morphological variation between ploidy levels and taxa associated in any way with differences in geographic distribution?

Materials and Methods

Crataegus L. is a genus of approximately 200 species of woody plants, part of a mostly fleshy-fruited clade (tribe Maleae Small) within an expanded subfamily Amygdaloideae Arn. (Rosaceae) (Potter et al. 2007; McNeill et al. 2012). *Crataegus* is taxonomically complex because, while the subgeneric classification of the genus into taxonomic sections and series (Phipps et al. 1990; Phipps and O'Kennon 2002) is broadly supported by morphology, not all subgeneric groups have been shown to be monophyletic (Lo et al. 2007, 2009). Moreover, species circumscriptions often inadequately reflect the occurrence of hybridization, gametophytic apomixis, and polyploidy. Black-fruited *Crataegus* series *Douglasianae* (Loud.) Rehder is one example of an apparently monophyletic group (Lo et al. 2007, 2009). This group is widespread in the Pacific Northwest of the United States and Canada and includes two well-known species, *Crataegus suksdorfii* (Sarg.) Kruschke and *C. douglasii* Lindl. *Crataegus suksdorfii* is restricted to the mesic conifer forest region of the Pacific Northwest (Brunsfeld and Johnson 1990), while *C. douglasii* can grow in more xeric areas and so has a much wider distribution, extending from the eastern slopes of the Cascades to the Rocky Mountains, with disjunct populations east of the continental divide (Hargrove and Luxmoore 1998; Dickinson et al. 2008).

Diploid, triploid, tetraploid, and pentaploid *C. suksdorfii* are now known (Dickinson et al. 2008; Lo et al. 2010a, 2013; Coughlan 2012), although only one of these cytotypes has, as yet, been given

taxonomic recognition. *Crataegus gaylussacia* A. Heller was described from Sonoma County, California, and has been shown to be triploid (Coughlan 2012, Dickinson et al. unpublished data), and to correspond more or less to the morphology of autotriploid individuals found elsewhere that would otherwise be referred to *C. suksdorfii* (Lo et al. 2010a). *Crataegus gaylussacia* has priority over the name *C. suksdorfii* if these names are applied to all 20-stamen, black-fruited hawthorns of the Pacific Northwest without regard to ploidy level (Phipps 2012; VASCAN 2013). In view of the variation in ploidy level documented for these plants (Talent and Dickinson 2005; Lo et al. 2013), we will retain the older usage of these names here (i.e., as referring to different biological entities). A publication clarifying the use of these names is currently in preparation (T. A. Dickinson, unpublished manuscript).

In contrast to *C. suksdorfii*, individuals of *C. douglasii* are almost exclusively tetraploid (Talent and Dickinson 2005; Lo et al. 2013), and distinctive morphotypes that have received taxonomic recognition (Phipps and O'Kennon 1998, 2002) appear to be of hybrid origin (Zarrei et al. 2012). Introgression from *C. douglasii* appears to be responsible for the allopolyploid cytotypes of *C. suksdorfii* (Lo et al. 2010a). The wider geographic distribution of tetraploid *C. douglasii* is associated with an apparently almost complete reliance on asexual seed production (Talent and Dickinson 2007; Coughlan 2012; Lo et al. 2013), as is also the case in polyploid *C. suksdorfii* (Dickinson et al. 1996; Lo et al. 2013). Diploid *C. suksdorfii*, on the other hand, appears to produce exclusively biparental, sexual seeds (Coughlan 2012; Lo et al. 2013).

Sampling

Sampling for this study draws on a large collection of mainly Pacific Northwest and Ontario specimens of *Crataegus* series *Douglasianae*, assembled and georeferenced for studies of morphological, breeding system, and ploidy level variation, that are housed for the most part in the Green Plant Herbarium (TRT) of the Royal Ontario Museum (Table 1). Many, but not all of these, are vouchers for studies of DNA sequence variation in the group (Lo et al. 2007, 2009; Coughlan 2012; Zarrei et al. 2012). To more fully sample the continental United States range of *C. douglasii*, permission was obtained to remove leaf material from two borrowed specimens from Michigan (Table 1). Specimens were selected that had sufficient mature leaves to permit removal of some for destructive sampling. In the geographic study, specimen selection had the further criterion that only *C. douglasii* and *C. suksdorfii* (both diploids and autotriploids) would be compared, unconfounded by being combined as in allopolyploid *C. suksdorfii* (Lo et al. 2009, 2010a). Locations of the individuals and populations studied were mapped using SimpleMappr (Shorthouse 2010).

Ploidy level determination. Most specimens studied are vouchers for flow cytometric ploidy level determinations (Talent and Dickinson 2005). However, because *C. douglasii sensu* lato has been found to be almost exclusively tetraploid (Talent and Dickinson 2005, Talent and Dickinson unpublished data), we have included some specimens of this taxon for which flow cytometric or chromosome count data are unavailable, and for our purposes have assumed that they are tetraploid (Table 1).

Stomatal Size – Population Level Comparison

Specimens for this study were selected to compare population samples of diploid (two populations, $n = 7$ and $n = 10$, Table 1), autotriploid, allotriploid, and allotetraploid (one population each, all $n = 10$) *C. suksdorfii* with two samples (each $n = 10$) of tetraploid *C. douglasii*, each of the latter sympatric with one of the allopolyploid *C. suksdorfii* populations Table 1 (Dickinson et al. 1996; Lo et al. 2009, 2010a).

Two leaves, randomly chosen from both short and long shoots were removed from each specimen, and two approximately 1 cm × 1 cm segments were excised from each leaf. For each leaf a random number from one to six was obtained and used to select a sampling position on the leaf (Fig. 1). Leaf segments were softened for 30–40 min in 70% ethanol, rinsed in tap water for 15–20 min, and then submerged overnight in 100% Drano® (S. C. Johnson & Son, Inc., Racine, WI) to clear the leaf segments (Buechler 2000). The following day, the samples were rinsed in water for 15–20 min, mounted in water on a microscope slide, and examined under Nomarski differential interference contrast at 800× magnification. Using MorphoSys v. 1.29 (Meacham and Duncan 1991), stomatal outlines were captured in order to calculate their length, width, area, and perimeter. In each leaf segment, five stomates were measured in five different quadrants of the leaf fragment: upper left, upper right, bottom left, bottom right, and middle, giving a total of 25 stomates measured per segment. To avoid counting a stomate more than once, the same counting sequence was used consistently (i.e., after the first stomate, go up, skip one, measure the following one; next, go left, skip one, count the following one; then up again and so on).

Stomatal Size and Density – Geographic Study

Specimens for this study were selected to compare diploid and autotriploid *C. suksdorfii*

TABLE 1. SITE AND VOUCHER DATA FOR POPULATION AND GEOGRAPHIC STUDIES OF CELL SIZE VARIATION IN RELATION TO PLOIDY LEVEL IN *CRATAEGUS* SERIES *DOUGLASIANAE* (ALL 2004- COLLECTIONS BY TAD, E. Y. Y. LO, AND S. NGUYEN; D1619 AT MT02 COLLECTED 2001 BY TAD, F. GERVAIS, AND J. S. DICKINSON). Sites are numbered as in Fig. 7, and labeled as in previous studies. Ploidy level determinations based on flow cytometry (Talent and Dickinson 2005; Lo et al. 2013). Except as noted, all vouchers are from the continental USA, and are housed at TRT. Some ploidy levels, where known, were determined by N. Talent from embryo (*), rather than leaf, tissue. Collector is TAD unless indicated otherwise (TAD = T. A. Dickinson; RED = R. E. Dotterer; EL = E. Y. Y. Lo; RML = R. M. Love).

State	Taxon	County	Site	Lat. N, Long. W	Elev. (m)	Site notes	Vouchers (population study)	Vouchers (geographic study)	Ploidy
California	*C. douglasii* Lindl. *sensu* lato	Plumas	(1) CAR1, Crescent Mills	40.094, 120.911	1068	within 120 m, and 11 m above, Indian Cr.		*RED-CAR036*	4x*
		Shasta	(2) CAR2, Dana	41.114, 121.567	1017	within 100–110 m of, and at about the same level as, Dana Cr.		*2006-12*	4x
	C. suksdorfii (Sarg.) Kruschke	Marin	(3) CA5, 1 km W of Tomales	38.247, 122.913	87	on banks of seasonal watercourses		*2010-5*	3x*
		Siskiyou	(4) CAR5, Scott Valley	41.397, 122.838	872	Scott R. floodplain		*2006-15*	2x
								2006-22	2x
		Sonoma	(5) CA7, Sebastopol	38.36, 122.8	180	Cunningham Marsh		*R. Thompson 564*	3x
								R. Thompson 565	3x
								R. Thompson 566	3x
Idaho	*C. douglasii* Lindl. *sensu* lato	Adams	(6) ID06, Last Chance Campground	44.989, 116.19	1425	within 10 m of Goose Cr.	*2005-05, 2005-06, 2005-07, 2005-08, 2005-11, EL-166, EL-179, EL-180, EL-181, EL-183*		4x
			(7) ID06a, Last Chance Road and Goose Creek	44.964, 116.206	1293	within 10 m of Goose Cr.		*2001-82*	4x*
		Idaho	(8) White Bird	45.758, 116.306	476	roadside, above Whitebird Cr.		*RML-9504*	4x*
		Lemhi	(9) ID16, Salmon River	45.367, 113.952	1130	within 70 m, and 7 m above, Salmon R.		*EL-190*	4x
		Nez Perce	(10) ID20, Highway 3	46.522, 116.733	291	within 300 m, and 5-10 m above, Little Potlatch Cr.		*EL-121*	4x
	C. suksdorfii (Sarg.) Kruschke	Adams	(11) ID06, Last Chance Campground	44.9989, 116.19	1425	within 10 m of Goose Cr.	*EL-162, EL-163, EL-164, EL-167, EL-168, EL-172, EL-173, EL-179, EL-182, EL-184*		3x

TABLE 1. CONTINUED.

State	Taxon	County	Site	Lat. N, Long. W	Elev. (m)	Site notes	Vouchers (population study)	Vouchers (geographic study)	Ploidy
Michigan	*C. douglasii* Lindl. *sensu* lato	Houghton	(12)	47.033, 88.617	200			*Richards 2575* (CM)	-
		Keweenaw	(13)	47.37, 88.303	330			*Richards s.n.* (DAO 591508)	-
Montana	*C. douglasii* Lindl. *sensu* lato	Lake	(14) MT06, Beaverhead Drive	47.486, 114.113	923	within 50 m, and less than 1 m above, Crow Cr.		*2001-31*	4x*
		Powell	(15) MT02, Kleinschmidt Flat	47.02, 112.946	1319	Cooper Lake Rd., within 50 m, and less than 1 m above, Rock Cr.	*D1611, EL-24, EL-31, EL-32, EL-34, EL-35, EL-38, EL-39, EL-40, EL-46*		4x
								EL-40	4x
	C. suksdorfii (Sarg.) Kruschke	Powell	(15) MT02, Kleinschmidt Flat	47.02, 112.946	1319	Cooper Lake Rd., within 50 m, and less than 1 m above, Rock Cr.	*D1619, EL-29, EL-30, EL-33, EL-36, EL-37, EL-41, EL-43, EL-44, EL-45*		4x
Oregon	*C. douglasii* Lindl. *sensu* lato	Grant	(16) Southfork Rd.	44.372, 119.547	836	within 50 m, and 5 m above, the South Fork, John Day R.		*RML C2003-20*	4x
		Multnomah	(17) Frontage road S of I-84 near Dodson	45.604, 122.04	29	within 300m, and 20 m above, the Columbia R.		*RML-8521*	-
		Umatilla	(18) Blue Mountains, S side of OR Hwy 204	45.795, 118.359	896	roadside within 100 m, and 17 m above, tributary of Pine Cr.		*2008-81*	4x
	C. suksdorfii (Sarg.) Kruschke	Columbia	(19) OR11, Sauvie Island	45.734, 122.767	4	beach ridge within approx. 30 m of the Columbia R.	*2005-16, EL-103, EL-104, EL-105, EL-106, EL-107, EL-109*		2x
			(19)			beach ridge within approx. 30 m of the Columbia R.		*P. Zika 18477*	2x
		Douglas	(20) OR04, Upper Elk Meadows Research Natural Area	43.527, 122.905	1240	open seep (Curtis 1986)		*RML C2003-35*	3x

TABLE 1. CONTINUED.

State	Taxon	County	Site	Lat. N, Long. W	Elev. (m)	Site notes	Vouchers (population study)	Vouchers (geographic study)	Ploidy
		Josephine	(21) OR20, Deer Creek Center	42.277, 123.647	387	banks of Deer Cr.		*2010-11*	2x
								2010-12	2x
		Lane	(22) OR06, Patterson Mountain Prairie	43.767, 122.619	1307	open seep	*EL-48, EL-49, EL-50, EL-51, EL-52, EL-53, EL-54, EL-56, EL-57, EL-63*		3x
			(22)					*EL53*	3x
			(22)					*EL56*	3x
		Linn	(23) OR01, Cogswell-Foster Reserve	44.334, 123.122	88	vernally moist Willamette Valley prairie, within 500 m and 2 m above, Little Muddy Cr. (Love and Feigen 1978)	*EL-68, EL-72, EL-75*		2x
			(23)					*RML-8712*	2x*
		Washington	(24) Hillsboro	45.557, 122.888	72	Edge of second-growth woods, powerline clearing		*P. Zika 18483*	2x
Washington	*C. douglasii* Lindl. *sensu* lato	Kittitas	(25) WA20, Ronald	47.24, 121.032	725	roadside hedgerow, within 100 m, and 5–10 m above, tributary of Cle Elum R.		*2005-21*	4x*
		Thurston	(26) WA21, Mound Prairie, E side of Scatter Creek Rest Area on Interstate 5	46.837, 122.984	65	dry disturbed meadow, within 900 m, and 6 m above, Scatter Cr.		*P. Zika 18438*	4x
	C. suksdorfii (Sarg.) Kruschke	Clark	(27) Ridgefield	45.855, 122.718	73	thickets at edge of meadow, within 500 m, and 70 m above, Mud Lake		*P. Zika 18486* (same individual as *P. Zika 18918*)	2x
								P. Zika 18918 (same individual as *P. Zika 18486*)	2x*
		Lewis	(28) Drews Prairie	46.467, 122.884	90	wet prairie remnant		*P. Zika 18995*	2x

TABLE 1. CONTINUED.

State	Taxon	County	Site	Lat. N, Long. W	Elev. (m)	Site notes	Vouchers (population study)	Vouchers (geographic study)	Ploidy
Wyoming	*C. douglasii* Lindl. *sensu lato*	Teton	(29) Targhee National Forest	43.277, 110.795	1782	Snake River floodplain		*Phipps 7459*	-
Ontario, Canada	*C. douglasii* Lindl. *sensu lato*	Thunder Bay District	(30) ON19, Pine River, first rapids	48.054, 89.507	211	Shrubby floodplain		*D1294*	4x*

with tetraploid *C. douglasii* across their continental United States ranges (Fig. 2, Dickinson et al. 2008). Leaf samples were examined from 15 *C. suksdorfii*, including eight diploids and seven autotriploids, and 17 tetraploid *C. douglasii* trees (Table 1).

The methods were the same as for the population-level study above, with the following exceptions. Softening the leaves was deemed unnecessary, and leaves were submerged in Drano® for 72 hr. Since the results of the previous study demonstrated that the sampling location on the leaf (Fig. 1) affected stomata size significantly (see Results), we restricted this study to leaf sections in the middle right of the leaf (section four in Fig. 1). Within each 1 cm^2 leaf section we examined an areole in each of four different, spatially separated regions (areoles are areas bounded by quarternary veins on all sides). We captured images of an areole at different focal depths using an Infinity 1 digital camera and the Infinity Analyze program supplied with it (Lumen*era* Corporation, Ottawa, ON). The images were stacked using Combine Z image software (Hadley 2010), and the stacked images were then imported back to the Infinity Analyze program. This was used to delimit individual areoles, determine their area, and count all the stomata found within an areole. We also used the program to measure the length and width of each stomata (Fig. 3). All measurements were automatically stored in an M.S. Access™ database (Microsoft, Redmond, WA).

Environmental data. Latitude and longitude for each collection site (Table 1) were used to obtain climate data (1971–2000 monthly normals for precipitation, and maximum and minimum temperature) using the online PRISM Data Explorer (PRISM Climate Group 2012). The geographic coordinates were also used to ascertain the elevation at each site using Google Earth (Version 7.1.2.2041 [software] available from http://www.google.com/earth/). Google Earth was also employed to estimate the proximity of sampling sites to bodies of water. The analyses described below used seasonal (quarterly) averages of the monthly normal values (Q1, winter, December–February; Q2, spring, March–May; Q3, summer, June–August; and Q4, fall, September–November). Some analyses also included the average minimum and maximum temperatures for the year, and total normal precipitation. Analyses of just the quarterly averages also included site elevation and latitude.

Statistical analyses. Statistical analyses were performed using R (R Development Core Team 2004). Normality of the size measurements was checked using qq plots and the Shapiro-Wilks test. Measurements found to be lognormally distributed were log-transformed. When analyz-

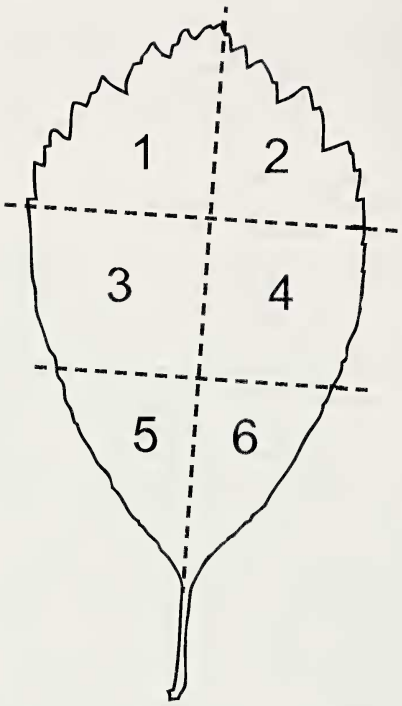

FIG. 1. Diagram of a *Crataegus* leaf divided into six sections. For the population-level study, the sampled section was chosen randomly while, for the geographic study, the mid-right section (4) was always used. In all cases the abaxial surface of the leaf was examined as shown here.

ing the stomata size data, two different methods were used to avoid the pseudoreplication problem of having many stomata measurements from the same leaves and trees. First, the mean values for each tree were calculated, and then compared. In the second analysis, we used a linear mixed effects model to determine the effect of ploidy and taxonomic group while accounting for the replication within leaves and trees. To analyze the relationship between stomatal traits, ploidy level, and climate variables we conducted a MANOVA and principal component analysis. We used the fourth-corner function in R from the ade4 package (Legendre et al. 1997, Dray and Legendre 2008) in order to infer relationships between our morphological data (stomata size and density; nuclear DNA content) and the environmental features of the sites referred to above.

RESULTS

While examining our *C. douglasii* and *C. suksdorfii* leaves, we made basic observations on the leaf surface characteristics (Fig. 3). The outlines of epidermal cells were wavy and cells surrounding the stomata appeared anomocytic. This is consistent with Metcalfe and Chalke's (1979) description of Rosaceae epidermis, but not with observations of other *Crataegus* species studied by Ganeva et al. (2009).

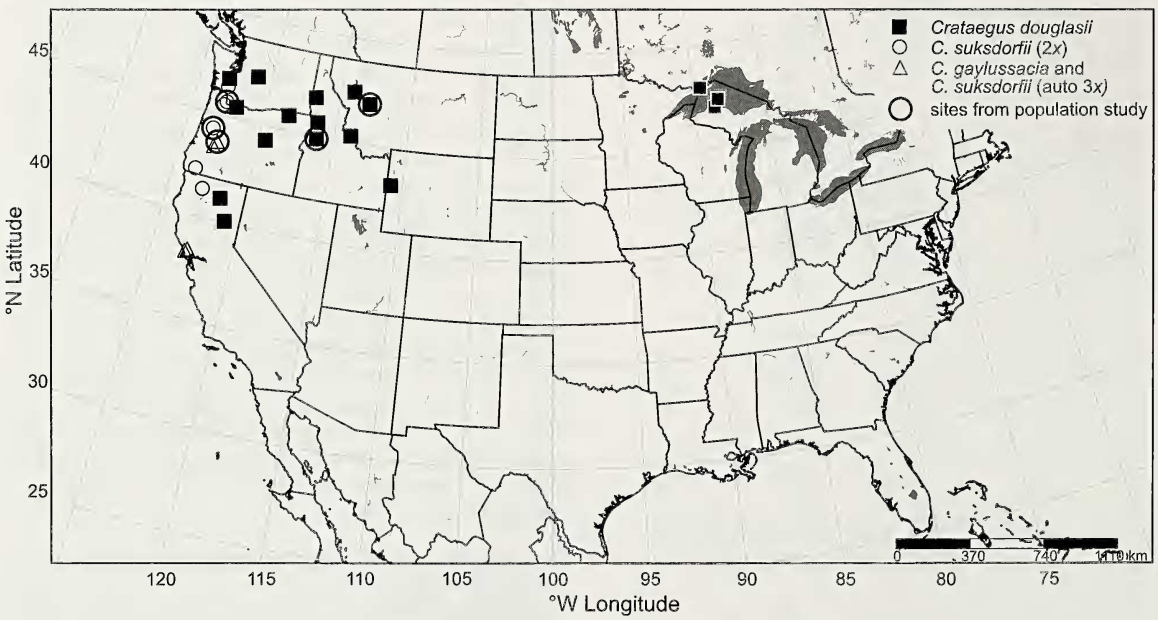

FIG. 2. Map showing sites where tetraploid *C. douglasii* (closed squares), diploid *C. suksdorfii* (open circles), and triploid *C. gaylussacia* and *C. suksdorfii* (open triangles) were collected for the geographic study (Table 1). Sites used in the population study are circled.

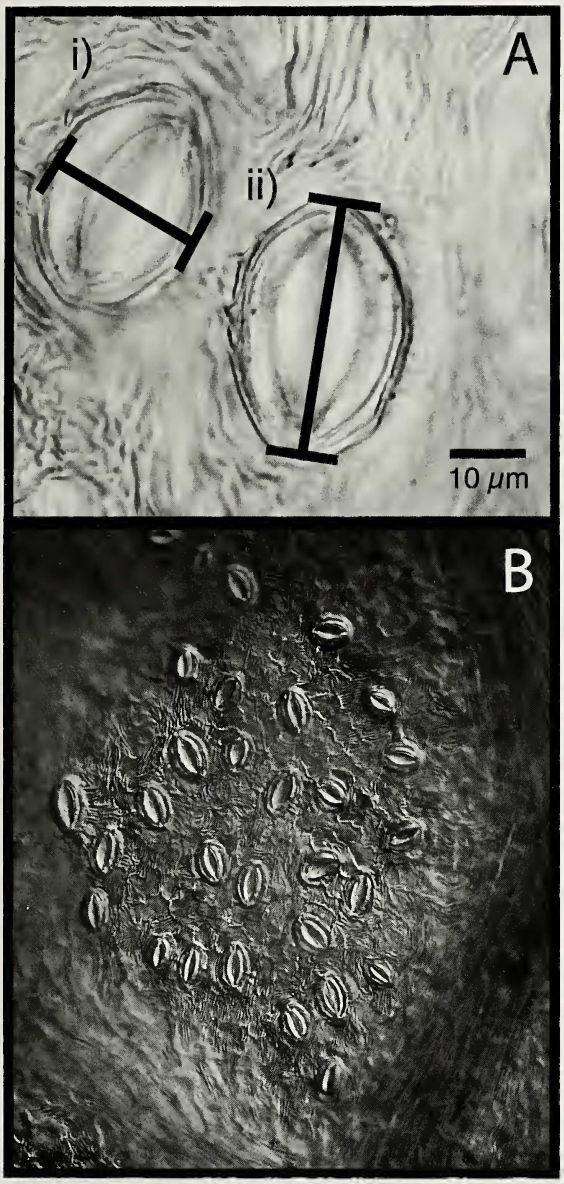

FIG. 3. Panels show *Crataegus douglasii* stomatal measurements: (A) Stomata width (i) and length (ii) were measured as the distances between the midpoints; (B) *Crataegus douglasii* stomata shown under 80× magnification. Stomata density was estimated by counting the number of stomata within an areole, and dividing by the total area of the areole.

Stomatal Size – Population-Level Study

Variation in stomatal size. The stomata ranged in length from 21 μm to 54 μm and their total areas ranged from 264 μm² to 1486 μm². As expected, stomata from tetraploid individuals were the largest (mean length 36 μm, compared to the overall mean of 34 μm). These results are consistent with analyses on the other size measures (width, perimeter, and area). Diploids had slightly larger average sizes than triploids, which were the smallest of the three ploidy groups. This is surprising, since triploids did have higher DNA content then diploids, which is expected to be correlated with cell size. We tested for such a relationship between DNA content and stomata size within each ploidy level, but there were no significant correlations (all $P > 0.05$).

The mean values for each leaf were determined and found to be normally distributed. Average stomata characteristics were consistently different between ploidy levels (all P values <0.001, see Fig. 4 for length), but not in a proportional manner (see below). Using an ANOVA to assess which factors affected stomatal characteristics, we found that both ploidy level and section of the leaf (Fig. 1) were significant (ploidy level and stomata length, $P < 0.001$; ploidy level and area, $P < 0.001$; leaf section and stomata length $P < 0.001$; leaf section and stomata area $P < 0.001$).

The mixed effects model of all the individual stomatal measurements was consistent with the analysis using size means. When examining the length of stomata, we found that ploidy was a significant factor ($P < 0.001$), and that the location measured on the leaf was as well ($P < 0.001$). In addition there was a significant interaction between ploidy level and measured location ($P = 0.0071$). The same was true when examining stomata area, with both ploidy level ($P < 0.001$) and leaf section ($P < 0.001$) being significant factors, the interaction term was also significant ($P = 0.0124$). Within tetraploids (*C. douglasii*, 4*x C. suksdorfii*; Fig. 4; Table 1), species was not a significant factor for stomata size (all P values >0.05).

Stomatal Size – Geographic Study

Variation in stomatal size. Stomata ranged in length from 15 μm to 57 μm, and the widths ranged from 9.8 μm to 38 μm. As was true in the population-level study, tetraploid *C. douglasii* had the largest stomata, and autotriploid *C. suksdorfii* had the smallest, although they were not significantly different from their diploid conspecifics (Fig. 5).

Again, two analyses were conducted to avoid pseudoreplication. Tree means were used to examine the effect of ploidy on stomata length and width. Ploidy level was a significant factor for both length and width. The second analysis, using the full data set while accounting for pseudoreplication within leaves and trees by including them as random factors, confirmed these findings, as the linear mixed effect models showed that ploidy was a significant factor for both stomata length ($P < 0.001$) and width ($P < 0.001$).

Variation in stomatal density. Stomatal density ranged from 88 stomates/mm² to 323 stomates/

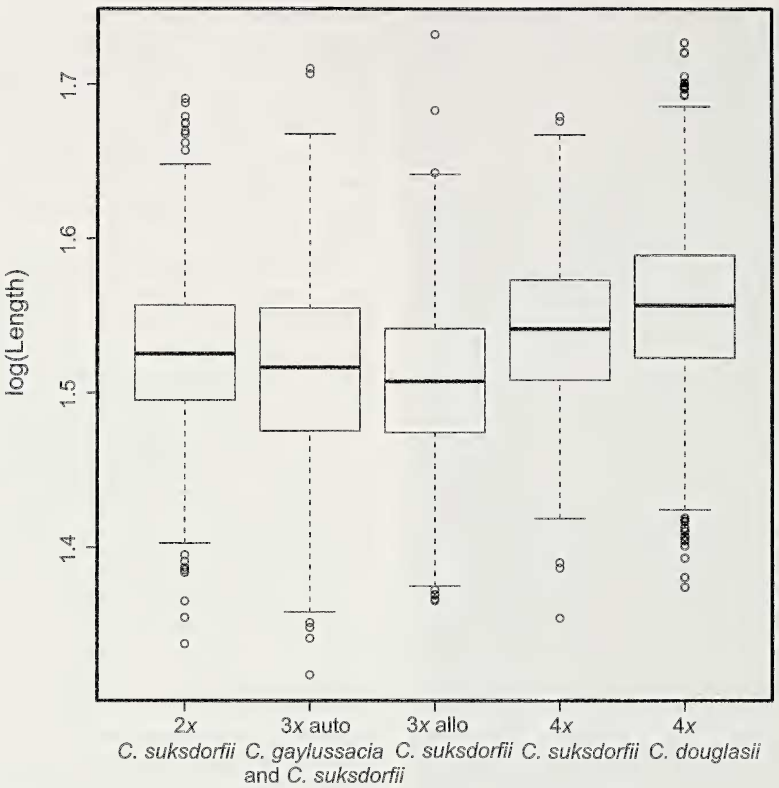

FIG. 4. Box plot of size (length) of stomata for *Crataegus* taxa in the population study. The dark middle lines show the median values, and two ends of the box show the 1st and 3rd quartiles. The whiskers show the interquartile range multiplied by 1.5. All values outside this range (outliers) are shown as open circles. Both ploidy level and section of the leaf (Fig. 1) were significant (ploidy level $P < 0.0001$; leaf section $P < 0.0001$).

mm^2 (Fig. 5A). Note that the densities are reported here in stomates/mm^2 for ease of interpretation, but the areoles were 0.16 mm^2 on average. The *C. douglasii* had an average stomata density of 166, while the diploid and triploid *C. suksdorfii* had average densities of 169 and 214 respectively. When all data were included in a simple ANOVA model, there was a significant difference between *C. douglasii* and *C. suksdorfii* ($P = 0.0215$), but this difference was driven by the higher density in triploids, so that when tree and leaf were included as random effects, the species difference was no longer significant ($P = 0.21$). Likewise, an ANOVA on tree means showed that there was not a significant difference in stomata density between ploidy levels ($P = 0.16$) or species ($P = 0.24$).

Environmental correlates. Comparisons between sites occupied by tetraploid *C. douglasii* and those occupied by *C. suksdorfii* (both diploids and autotriploids) showed the former to be cooler and more xeric (Figs. 6, 7). It is important to note that the environmental data were atmospheric, and may not be a precise reflection of water relations on the ground at each site. Nevertheless, except for the two Michigan sites for which hydrological conditions are unknown, each of the samples studied here are on flood plains, adjacent lakeshores, or on slopes down which water moves at or below the surface in response to local atmospheric conditions (Table 1). The results of the MANOVA analysis demonstrated a significant relationship between three climatological variables (annual precipitation, minimum and maximum temperature) and the *Crataegus* species at a site ($P = 0.0058$). Fourth-corner analyses demonstrated a significant relationship between several environmental factors and some but not all of the features of the hawthorn stomata (Fig. 8). Stomatal length and width covaried significantly with latitude, precipitation, and temperature, although the direction of the relationship depended on the quarter of the year examined (Fig. 8). Ploidy level was positively associated with elevation and negatively associated with precipitation and temperature (Fig. 8). There were no significant relationships between stomatal density and any of the environmental factors (Fig. 8).

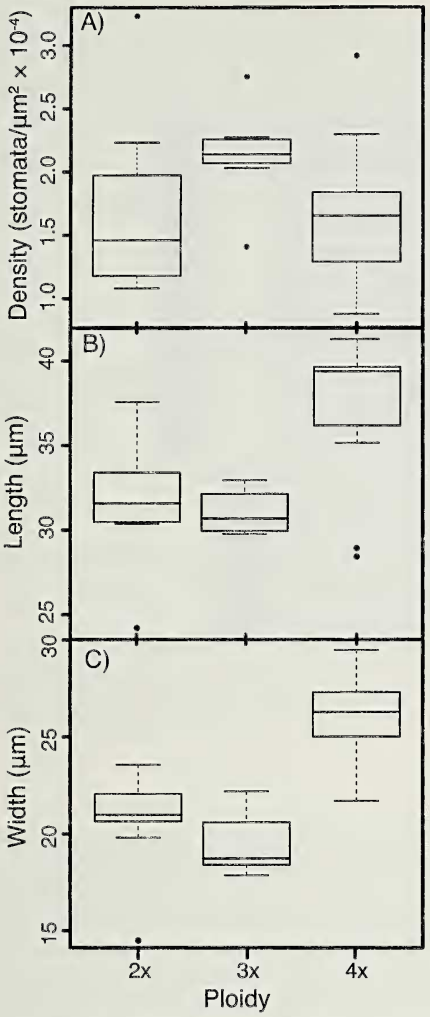

FIG. 5. Boxplot of stomata characters of diploid (2x) and autotriploid (3x) *C. suksdorfii* and tetraploid (4x) *C. douglasii* in the geographic study: (A) Density; (B) Length (μm); (C) Width (μm). The dark middle lines show the median values, and two ends of the box show the 1st and 3rd quartiles. The whiskers show the interquartile range multiplied by 1.5. All values outside this range (outliers) are shown as dark circles.

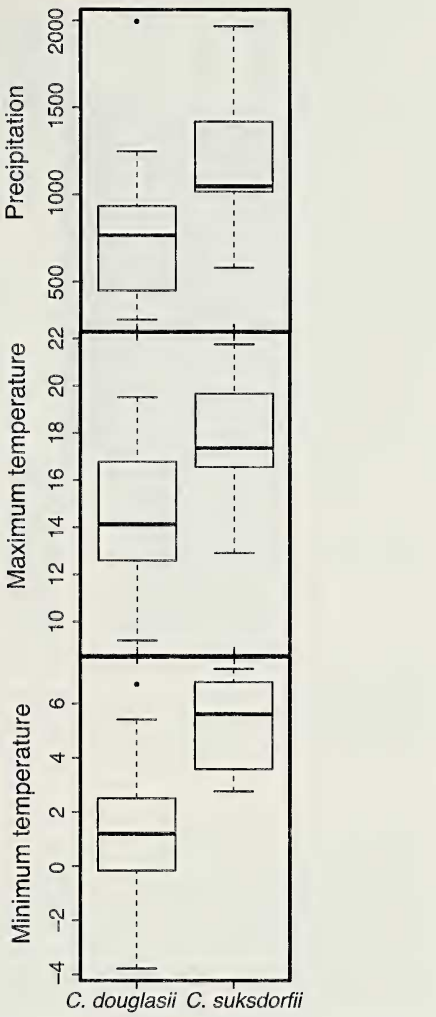

FIG. 6. Boxplot of climate summaries comparing the *C. douglasii* and *C. suksdorfii* sites (diploid and autotriploids pooled) used in the geographic study. Precipitation is the annual total (mm), maximum and minimum temperatures are annual averages. The dark middle lines show the median values, and two ends of the box show the 1st and 3rd quartiles. The whiskers show the interquartile range multiplied by 1.5. All values outside this range (outliers) are shown as dark circles.

Predicting Ploidy Level from Stomatal Measurements

Data from the population-level study and the geographical one were pooled in order to depict the predictive relationship between nuclear DNA content and stomatal size. Plotting nuclear DNA content against stomatal length (Fig. 9) demonstrates the way in which variation at the levels of leaf, individual, and sampling site vitiates any attempt to predict ploidy level from stomatal size: the ranges in size for each ploidy level overlap so much that any given size except the very smallest or the largest might correspond any one of the three ploidy levels studied here (Fig. 9). Similar relationships were observed by Marshall (1978) in Manitoba *Crataegus chrysocarpa* and *C. succulenta*. Although Marshall was probably incorrect in his inference that the smaller stomata of *C. succulenta* meant that this taxon is diploid (Ontario specimens have been shown to be triploid; Talent and Dickinson 2005), their size relative to those of *C. chrysocarpa* (tetraploid; Talent and Dickinson 2005) demonstrates the same trend we have observed.

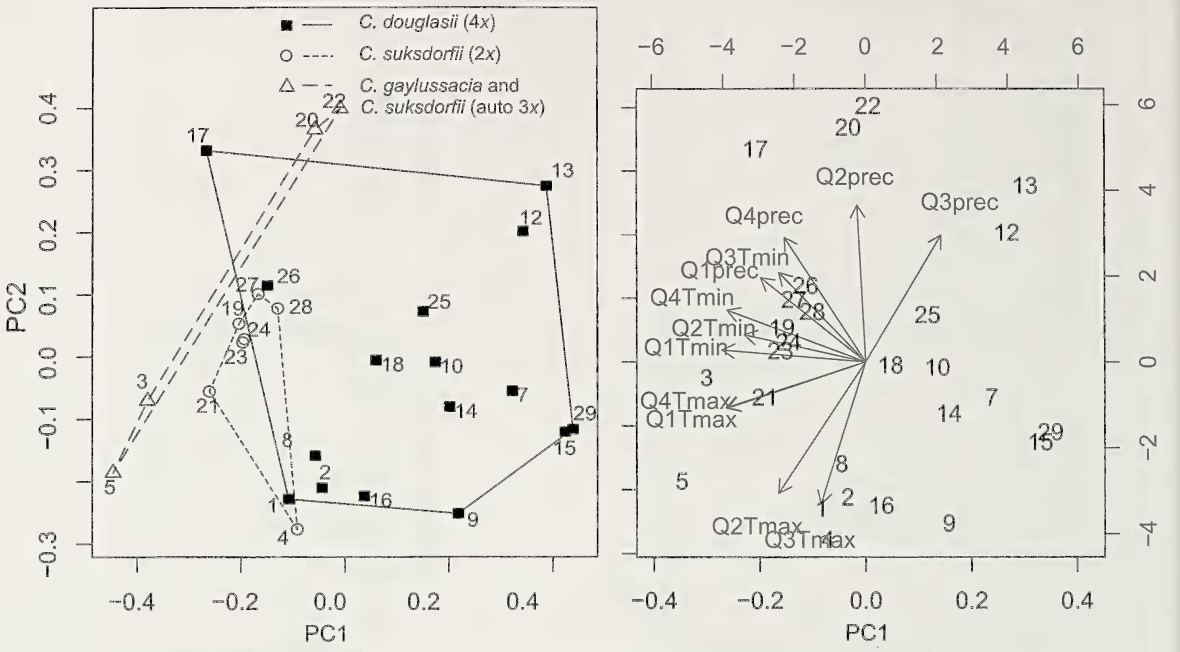

FIG. 7. Principal component analysis of quarterly climate data of the *Crataegus* sites used in the geographic study (numbered as in Table 1). The four quarters are winter (Q1), spring (Q2), summer (Q3), and fall (Q4). Left panel: convex hulls enclose sites grouped by taxon and ploidy level present at each one. Right panel: PCA biplot with vectors representing contributions of quarterly climate variables.

DISCUSSION

The taxonomic complexity of the genus *Crataegus* has long been recognized (Camp 1942), as has the connection between this complexity and the occurrence of gametophytic apomixis, hybridization, and polyploidy (Dickinson and Phipps 1985, 1986; Lo et al. 2010b). This connection makes knowing the ploidy level and breeding system critical in order to make clear taxonomic judgments regarding *Crataegus* species. Determining ploidy level from chromosome counts is difficult because the tissues in which meiotic metaphases can be accumulated are available for less than a week, annually, at any given locality. Flow cytometry is an extremely valuable tool when studying *Crataegus*, but this method works mainly on living tissue, making it nearly useless especially with older herbarium material (e.g., type specimens). The only exception to this is the ability to obtain flow cytometric data from seeds (Table 1; Talent and Dickinson 2007). This approach has been shown to work on decades-old seeds, provided they have been stored in the cold (Talent personal communication). The ability to use a phenotypic trait like stomata size as an indicator of ploidy level would greatly simplify the study of this complicated genus. Unfortunately, based on our results, variation in this trait is not consistently related to ploidy level.

Although the largest stomata in our study were from the tetraploid *C. douglasii* trees, stomata from autotriploid *C. suksdorfii* individuals were generally smaller than those of diploids (Fig. 5). In addition, there was wide variation in size within a ploidy level, and too much overlap between ploidy levels to make a comparison of average stomata size useful for assessing ploidy (Fig. 9). The relationship between genome and cell size is weaker in trees than in other plants (Beaulieu et al. 2008). The lack of a difference between *C. suksdorfii* cytotypes in stomata size may be a reflection of this. Stomata size is not solely controlled by genome size; environmental or physiological factors may select for smaller cell size, or specifically for smaller stomata. Note that we deliberately excluded allopolyploid *C. suksdorfii* from the geographic comparison in order to focus on the contrast between diploid and autotriploid *C. suksdorfii* and tetraploid *C. douglasii* (Fig. 5).

There seemed to be more of a taxonomic grouping with respect to stomata size than a difference between ploidy levels (Fig. 5). The difference in stomata size between *C. douglasii* and *C. suksdorfii* could prove useful in determining the identity of previously collected specimens. This might be of use in the case of sterile specimens, where per flower stamen numbers are unavailable. Other factors affect stomata size, and the relationship between environmental

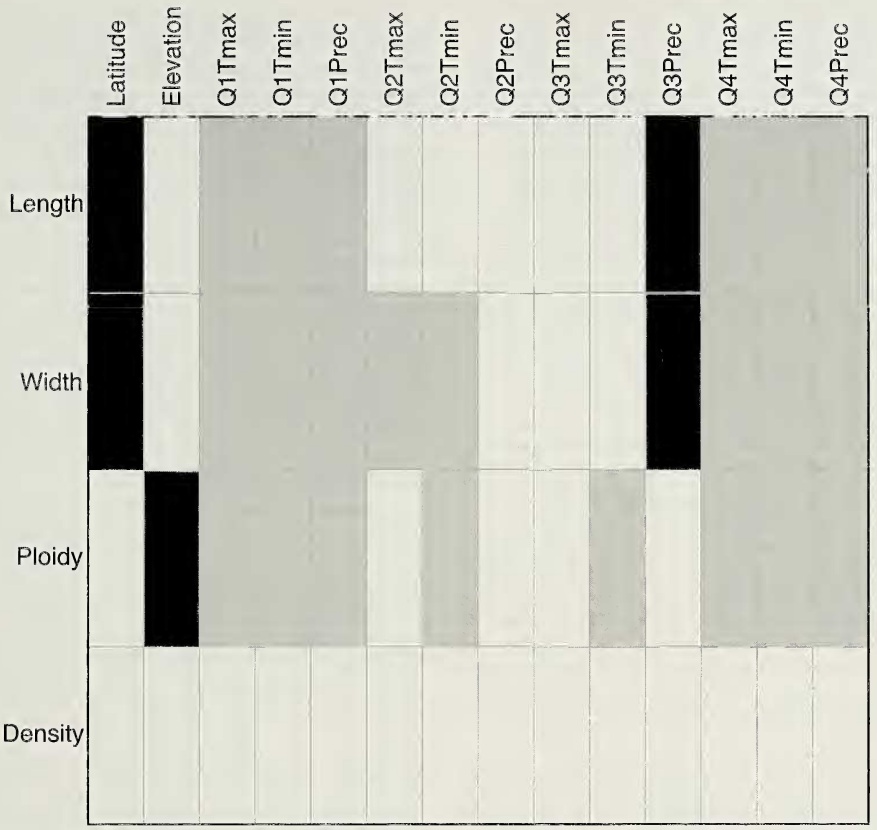

FIG. 8. Associations between *Crataegus* stomatal characteristics and ploidy level, and the geographic and climatic variables at the sites from which they were collected, as calculated by a fourth-corner analysis (Legendre et al. 1997). Significant positive associations are shown in black, significant negative associations are in grey and non-significant associations are in white. Latitude values were collated from the herbarium specimens used in the geographic study. Elevation values were gathered from Google Earth. Monthy averages for maximum temperature, minimum temperature, and precipitation were acquired using the PRISM database and used to calculate quarterly values. The four quarters are winter (Q1), spring (Q2), summer (Q3), and fall (Q4).

characteristics and stomata traits illustrate such an example. For *C. douglasii* and *C. suksdorfii*, spring and summer precipitation averages are positively correlated with stomata size (Figs. 7, 8). There are a limited number of triploid sites, and there is much environmental heterogeneity between those in California (presumptively autotriploid *C. gaylussacia*) and those in Oregon (autotriploid *C. suksdorfii*). However, these sites are representative of the known, and quite limited, distribution of *C. gaylussacia* on the one hand, and of autotriploid *C. suksdorfii* on the other.

Despite large strides in polyploidy research, we still know very little about the ecological implications of polyploidy (Soltis et al. 2010; Manzaneda et al. 2012). Past researchers have hypothesized that genome duplication confers advantages that lead to an increased or novel range for the polyploid species. However, little empirical work has been done to test this idea (Soltis et al. 2010). Stebbins addressed the uncertainty surrounding the effect of polyploidy on species ranges decades ago and suggested that clarification could be gained by carefully studying the morphological traits and habitats of increasing numbers of species groups (Stebbins 1950). In the years since, many different taxonomic groups have been examined. This research has demonstrated that polyploids often tolerate more extreme conditions than their diploid relatives (Lewis 1980), and one ecological difference that does seem common across many taxa is that plants with higher ploidy levels are found in more xeric conditions than their diploid relatives (Levin 2002). In our study group, tetraploid *C. douglasii* is found in more xeric conditions (in terms of mean annual precipitation) than diploid and triploid *C. suksdorfii* (Fig. 6). This is consistent with studies on other plants, where taxa with higher ploidy levels grow in drier conditions than their lower ploidy-level relatives, including in *Achillea* L. (Ramsey 2011), *Chamerion* (Raf.) Raf. ex Holub (Maherali et al. 2009),

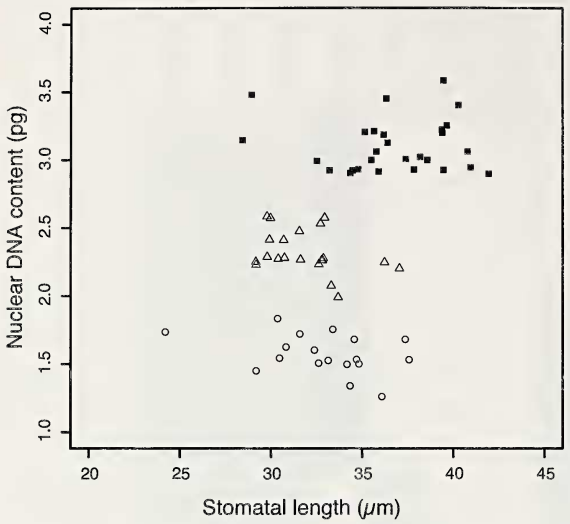

FIG. 9. Nuclear DNA content and stomatal length plotted to show why stomatal size cannot be used as a predictor of ploidy level given the variation between leaves, individuals, and sites using data from the combined population level (leaf region 4 only) and geographic samples. Tetraploid *C. douglasii*, filled squares. Open symbols, *C. suksdorfii*: circles, diploid; triangles, triploid.

and *Brachypodium* P. Beauv. (Manzaneda et al. 2012). Sites at which *C. douglasii* occurs are also, on the average, colder than those occupied by *C. suksdorfii* (Fig. 6). When the climate data are broken down into quarterly values, we see that while there is some overlap between the environments in which diploid *C. suksdorfii* and tetraploid *C. douglasii* occur, the tetraploid occupies a much wider range of environments than does the diploid (Fig. 7). Combined with the variation in breeding systems, this pattern of apomictic polyploids distributed over a wider range of environments than closely related sexual diploids (hence exhibiting a larger geographic range; Fig. 2) is what is referred to as geographical parthenogenesis (Hörandl 2006; Lo et al. 2013).

As seen in our study, a higher ploidy level tends to lead to larger cells and stomata. In many taxa this increase in stomata size is accompanied by a significant decrease in stomata density. Nevertheless, by using the fourth-corner method to infer the association between nuclear DNA content and features of stomata, and quarterly climate parameters and elevation, we have shown that stomata density does not vary significantly in relation to any of the environmental factors we examined (Fig. 8). In other groups it does not seem advantageous to have larger stomata in more xeric conditions, but in many cases, the decreased density more than compensates, so that the total pore space on a leaf is lower in higher cytotypes (Levin 2002). Polyploidy may confer other advantages on plants that enable them to tolerate dry environments. Polyploids tend to grow more slowly than diploid congenerics, which could be an advantage when resources are scarce (Grant 1981; Levin 1983; Deng et al. 2011). In addition, some polyploids have increased leaf water content and transpiration efficiency (Chen and Tang 1945; Tal and Gardi 1976). In *Crataegus*, allopolyploid *C. suksdorfii* occurs in drier areas, where no diploid populations occur. Perhaps this is due to the contribution from the *C. douglasii* genomes in these individuals. It is also important to consider effects separate from cytotype; genetic divergence driven by natural selection will occur between lineages after a polyploidization event. More research on the magnitude and mechanism for increased tolerance to dry environments in polyploids is needed.

As noted above, environmental descriptors like "drier" or "wetter," not to mention the precipitation data used in our analyses, relate to atmospheric conditions. In fact all of our sites except possibly Hillsboro, Washington, and the two Michigan ones, are within a kilometer (usually much less) of at least seasonally running or standing water, and the topography of many of these sites is such that ground water likely flows through the root zone most or all of the year (Table 1; e.g., Curtis 1986). Hawthorns are frequently found in riparian zones and this fact may help to explain the lack of correlation between stomatal size and growing season precipitation (Fig. 8). Figure 8 also documents the way in which diploid *C. suksdorfii* and autotriploid *C. gaylussacia* are restricted to a region of Mediterranean climate (high winter precipitation, summer drought) where stomatal size is negatively correlated with Q2 temperatures, and positively correlated with Q3 precipitation.

CONCLUSIONS

The interplay between cytotype, physiology and ecology is complex. Our study demonstrates that neither stomatal density nor stomatal size can be used to determine ploidy level in the *Crataegus* series *Douglasianae*, However, stomata size may be useful in differentiating between *C. douglasii* and *C. suksdorfii*. The two species occur in distinctive environments, and some climatological (precipitation, temperature) and geographic traits (latitude) are significantly related to stomata size. Polyploidy is a unique mechanism of speciation in its immediacy, and its propensity to cause speciation in sympatry. The ecological implications of polyploidization continue to be an important avenue of research.

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